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Resistance of *Venturia inaequalis* to demethylation inhibiting (DMI) fungicides

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Abstract

Sensitivity of *Venturia inaequalis* to demethylation-inhibiting (DMI) fungicides was investigated in field and laboratory conditions. Field experiments were conducted during 2008–2009, and the efficacy of triazoles – difenoconazole, flusilazole was established and compared with captan attributed to the *Phtalamide* chemical group. Sensitivities of monosporial isolates of *V. inaequalis,* originated from locations with different history of fungicide usage, were tested in laboratory. Mycellia growth inhibition on growth medium with flusilazole, difenoconazole and captan was monitored and susceptibility parameters (EC_{50} values) were determined using probit analysis. The isolates sensitivity *in vivo* was tested on apple seedlings using inoculation method.

In field experiments, low efficacy of both DMI fungicides, difenoconazole and flusilazole and high efficacy of multi-site fungicide captan were established. The EC_{50} values were ranging from 0.005–0.148 and 0.016–0.362 μg ml^{-1} for flusilazole and difenoconazole, respectively. Isolates originated from orchard where DMI fungicides had never been used, showed a normal susceptibility to all fungicides while isolates originated from experimental and commercial orchard showed resistance to both flusilazole and difenoconazole. All tested isolates showed a similar sensitivity to captan.

Key words: *Venturia inaequalis*, flusilazole, difenoconazole, efficacy, sensitivity, resistance.

Introduction

Apple scab, caused by *Venturia inaequalis* Cke. (Wint.), is the most economically important disease of apples worldwide. The disease primarily attacks leaves, flowers and fruits. If scab control measures are not taken well, losses from the disease may be 70% or more of the total fruit value (Agrios, 2005).

Fungicide application remains the primary tool for managing this disease. Sprays are routinely applied from bud burst at 7–10 day intervals until the risk of scab ceases. Protectant fungicides are used early in the season when there are only a few leaves or when infection period can be forecasted. Curative fungicides are used when a protectant fungicide applied before the infection was washed off by rain, a proctectant fungicide was not applied prior to the infection period, or the risk of primary infection was very high (Jobin, Carisse, 2007).

Demethylation-inhibitors compose one of the most important groups because of their curative action against scab and their excellent control of powdery mildew, the next most important apple disease in Serbia. Demethylation-inhibitors specifically target demethylation process at C-14α position of 24 methylenedihydrolanosterol and disrupt fungal sterol biosynthesis (Scheinpflug, Kuck, 1987; Köller, 1988). At the beginning of usage of the fungicides from this group, in the early 1980s, excellent control of many fungal diseases including apple scab was noted. However, their frequent and improper use has lead to a reduction of fungal population sensitivity, detected in some countries (Stanis, Jones, 1985; Hildebrand et al., 1988; Köller et al., 1997; Jobin, Carisse, 2007).

Resistance mechanisms to DMIs include overexpression of the CYP51A1 gene from *V. inae-*

qualis (Schnabel, Jones, 2000), as well as efflux mechanism (Nakaune et al., 1998) and point mutations (Delye et al., 1997).

The two common DMI fungicides used for apple scab control in Serbia are flusilazole (Olymp 10 EW, "Du Pont") and difenoconazole (Score 250 EC, "Syngenta"). However, over the last several years, a decreased efficacy of those fungicides in scab control has been reported, which is usually attributed to extended spray intervals and/or poor absorption due to unfavourable weather conditions.

The aim of this study was to determine the sensitivity of *V. inaequalis* population, previously exposed to DMI fungicides and population originated from location where those fungicides have never been used. Because of the importance of cross-resistance in antiresistance strategies, the present study also investigated cross-resistance between two members of DMI fungicide group.

Materials and methods

Efficacy trials. The trials were conducted in a research apple orchard (cv. 'Idared') on the property of the Faculty of Agriculture, Experimental Station Radmilovac during the seasons 2008–2009. The trials were arranged in a randomized complete block design with four replications, according to EPPO methods (EPPO, 1997). Individual treatment-block consisted of five trees. Treatments were carried out using motorized knap-sack sprayer ("Solo Port 423", Germany) by thoroughly wetting the trees (water volume: 1000 l ha^{-1}). Intervals between treatments were 7–9 days starting from tight cluster stage. The assessment was made on 200 leaves of long shoots and rosettes per plot. Infected leaves were ranged according to a scale: 0 = no attack, 1 = 1–3 spots per leaf, 2 = >3 spots per leaf. The disease severity was evaluated using the Townsend-Heuberger's formula (Townsend, Heuberger, 1943).

$$DS\ (\%) = \frac{\Sigma\ (nv)}{NV}\ x\ 100,$$

where n – degree of infection according to the scale,

v – number of leaves per category,

V – total number of leaves screened,

N – highest degree of infection.

The fungicide efficacy was calculated using Abbott's formula (Abbott, 1925).

$$Ef.\ (\%) = \frac{X - Y}{X}\ x\ 100,$$

where X – disease severity of the control,

Y – disease severity of the treatment.

Mean percent disease values from four replicate plots per treatment were calculated after disease ratings done on May 20, 2008 and June 15, 2009. The data were analysed separately for each trial using *Anova* and the means were separated by Duncan's multiple range test.

Fungicides. Commercial formulations of flusilazole (Olymp 10 EW, "Du Pont"), difenoconazole (Score 250 EC, "Syngenta") and captan (Merpan 80 WDG, "Makhteshim") at recommend doses for commercial usage were used in this study.

Isolates. To have as wide a range of fungal sensitivity to DMI fungicides as possible, samples were obtained from orchards with different histories of DMI fungicide usage. Scab infected apple leaves were collected during 2008 from three apple orchards in Serbia. The first orchard was an experimental orchard (Faculty of Agriculture, Experimental Station Radmilovac). The second orchard was Juzni Banat, Bela Crkva, the biggest commercial apple orchard in Serbia, both with a long DMI fungicide usage history. In the third orchard, positioned in the area of Zlatibor mountain, DMI fungicides had never been used (Table 1).

Table 1. Origin and fungicide history of the *V. inaequalis* isolates used

Year	Radmilovac (experimental orchard)		Bela Crkva (commercial orchard)		Zlatibor (untreated orchard)	
	DMI	total	DMI	total	DMI	total
2000	6	11	7	11	0	0
2001	8	13	8	12	0	0
2002	7	11	7	10	0	0
2003	6	9	8	11	0	0
2004	6	10	6	9	0	0
2005	8	11	8	12	0	0
2006	7	10	6	9	0	0
2007	7	9	8	10	0	0

Note. DMI – demethylation-inhibiting fungicides.

The leaves were placed between layers of paper and stored in a freezer at 4°C to dry. After two weeks, scab lesions with conidia were immersed in 1 ml of sterile distilled water and the suspension was spread on potato dextrose agar (PDA) and incubated at 15°C. After incubation for 24 h, germinated conidia were individually picked up with a sterile needle under microscopic observation and transferred to PDA containing antibiotic (tetracyclin 10 mg liter^{-1}). A total of 12 monosporial isolates of *V. inaequalis* were tested in this study (Table 2).

Table 2. List of isolates of *V. inaequalis* and their origins

Isolate code	Origin of isolate
$ViRM_1$	Experimental orchard Radmilovac, Belgrade
$ViRM_2$	Experimental orchard Radmilovac, Belgrade
$ViRM_3$	Experimental orchard Radmilovac, Belgrade
$ViRM_4$	Experimental orchard Radmilovac, Belgrade
$ViRM_5$	Experimental orchard Radmilovac, Belgrade
$ViBC_1$	Commercial orchard Juzni Banat, Bela Crkva
$ViBC_2$	Commercial orchard Juzni Banat, Bela Crkva
$ViBC_3$	Commercial orchard Juzni Banat, Bela Crkva
$ViBC_4$	Commercial orchard Juzni Banat, Bela Crkva
$ViBC_5$	Commercial orchard Juzni Banat, Bela Crkva
$ViZB_1$	Untreated orchard, Mountain of Zlatibor
$ViZB_2$	Untreated orchard, Mountain of Zlatibor

In vitro sensitivity tests. Sensitivities of *V. inaequalis* isolates to flusilazole, difenoconazole and captan were evaluated. Commercial formulations of fungicides were dissolved in water and incorporated into the autoclaved PDA medium which had been cooled to 55°C. Mycelia plugs (5 mm diameter) were removed with a cork borer from colony margins on the fungicide-amended and unamended PDA medium and incubated at 19°C. Flusilazole concentrations of 0.0, 0.001, 0.005, 0.01, 0.025, 0.05, 0.1 and 0.2 µg ml^{-1}, difenoconazole concentrations of 0.0, 0.005, 0.01, 0.025, 0.05, 0.1, 0.5 and 1 µg ml^{-1} and captan concentrations of 0.0, 0.5, 2.5, 5.0, 25.0, and 50.0 µg ml^{-1} were prepared. Five Petri-dishes (50 mm diameter) were used for each combination of concentration, isolate and fungicide. After 6 weeks of incubation mycelia radial growth was measured.

The mean colony diameter was measured (minus the diameter of the plug) and expressed as a percentage of the mean diameter of the control (unamended PDA). The EC_{50} values (defined as the fungicide concentration in ppm a.i. at which 50% of the radial growth was inhibited) were calculated using probit analysis (Finney, 1971). The resistance factor (RF) of each isolate for each fungicide was calculated by dividing the EC_{50} value of the isolate by the EC_{50} value of the most sensitive isolate.

In vivo sensitivity tests. An isolate which expressed the most sensitivity ($ViZB_1$) and isolate which showed the highest RF factor to both DMI fungicides ($ViBC_1$) in *in vitro* experiments were used in this study. *In vivo* sensitivities to flusilazole, difenoconazole and captan were tested on 6-week-old apple seedlings raised from Golden Delicious seeds (Sierotzki, List, 2006). Six seedlings were treated per fungicide concentration. Each set of seedlings was treated with 30 ml of fungicide solution of the following concentration of flusilazole: 20, 35, 50 and 80 mg l^{-1}; difenoconazole: 30, 45, 57 and 100 mg l^{-1} and captan: 1000 and 1500 mg l^{-1}. Inoculation was carried out using 30 ml of spore suspension for each isolate separately (2 x 10^5 spores per ml) using an air brush. The seedlings were then covered with nylon cloth and incubated in the climatic chamber for 48 h at 95% relative humidity and 16°C. After two days, the seedlings were transferred into a greenhouse at 20°C and 60–70% relative humidity with a photoperiod of 14 h day^{-1}. Disease severity was determined 20 days after inoculation by visually assessing the percentages of leaf areas infected and fungicide efficacy was calculated as previously described for efficacy trials.

Results and discussion

Field experiments. During 2008 and 2009, environmental factors were favourable for the development of infection caused by *V. inaequalis*, which resulted in 43.3% disease severity in the first, and 47.3% in the second year (Table 3). In 2008, the trees in the experiment received eight while in 2009, they received nine treatments. There were 7–9

days between treatments; fungicide treatments were aimed to prevent primary infections, usually before the favourable conditions for infections occurred, to achieve maximum efficacy of the fungicides.

During 2008, flusilazole did not show good efficacy (48.2%), while that of difenoconazole was 65.7%. Although the efficacies of the two DMI fungicides differed significantly ($P = 0.05$), neither of them showed a satisfactory efficacy. In the second experimental year, the efficacy observed was even lower than in the first year, for both fungicides. The efficacy of flusilazole (36.2%) was not significantly different than that of difenoconazole (38.9%). In Radmilovac, DMI fungicides have been used for several consecutive years with 6–8 treatments per year, which was more than one half of all of the fungicide treatments used at that location to control apple diseases. Considering the findings of many researchers, as well as the fact that according to FRAC classification, DMI fungicides belong to the group of fungicides that present a high risk for resistance development, the obtained results could be explained by multi-year use at the particular location (Gisi et al., 2000; Russell, 2004; Brent, Hollomon, 2007). On the other hand, the efficacy of captan, a fungicide with protective action, was very high in both years (89.8% and 95.3%, respectively). Short intervals among treatments, and the use before favourable conditions for infections occurred, resulted in good efficacy of this fungicide, which has non-specific mode of action and to which, despite a long use, fungal pathogens rarely develop resistance.

Table 3. Disease severity on apple leaves and efficacy of fungicides

Fungicide	Rate g a.i. ha^{-1}	Year			
		2008		2009	
		disease severity %	efficacy %	disease severity %	efficacy %
Control	–	43.3 a*	–	47.3 a*	–
Flusilazole	24	22.4 b	48.2	30.2 b	36.2
Difenoconazole	50	14.9 c	65.7	28.9 b	38.9
Captan	1000	2.0 d	95.3	4.8 c	89.8
LSD_{05}		3.25		1.74	

Note. * – values followed by the same letter do not differ significantly; $P = 0.05$, according to the Duncan's test.

In vitro tests. Isolates from Zlatibor ($ViZB_1$ and $ViZB_2$), where DMI fungicides have never been used, showed the greatest sensitivity to both examined demethylation inhibitor fungicides (Table 4). EC_{50} values recorded for flusilazole for these isolates were 0.005 μg ml^{-1} and 0.007 μg ml^{-1}, respectively, and EC_{50} recorded for difenoconazole were 0.016 μg ml^{-1} and 0.019 μg ml^{-1} for the two isolates, respectively. These results were similar to those obtained by Smith et al. (1991) in New York State for sensitivity of *V. inaequalis* to flusilazole and lower than the results obtained by Sholberg and Haag (1993) and Kunz et al. (1997) for sensitivity of *V. inaequalis* to flusilazole in Canada and Germany, respectively. All the examined isolates from locations where flusilazole and difenoconazole, and other DMI fungicides have been used for many consecutive years, showed significantly less sensitivity to the examined fungicides than isolates collected from the locations where those fungicides were not used. EC_{50} values for flusilazole used on isolates from the experimental orchard in Radmilovac, ranged from 0.031 μg ml^{-1} ($ViRM_5$) to 0.110 μg ml^{-1} ($ViRM_3$), (RF = 6.2–22) while EC_{50} for difenoconazole used on the same isolates ranged from 0.091 μg ml^{-1} ($ViRM_5$) to 0.209 μg ml^{-1} ($ViRM_3$), (RF = 5.7–13.1). Isolates from commercial orchard Bela Crkva, also showed significantly less sensitivity to both examined DMI fungicides. EC_{50} for flusilazole ranged from 0.038 μg ml^{-1} ($ViBC_5$) to 0.148 μg ml^{-1} ($ViBC_1$) with RF factors of 7.8 and 29.6, respectively. EC_{50} values for difenoconazole were 0.081 μg ml^{-1} ($ViBC_1$), (RF = 5.1), and 0.362 μg ml^{-1} ($ViBC_5$), (RF = 22.6).

Isolates that showed the greatest EC_{50} for flusilazole, also showed the lowest sensitivity to difenoconazole, which confirms cross-resistance and common genetic background for development of resistance in *V. inaequalis* to various demethylation inhibitor fungicides.

EC_{50} values for captan were very similar in all the tested isolates, regardless whether they were collected at the locations that were treated for many consecutive years with DMI fungicides or at locations where DMI fungicides had not been used.

Table 4. Sensitivity of *V. inaequalis* isolates to flusilazole, difenoconazole and captan

Isolate	Flusilazole		Difenoconazole		Captan	
	EC_{50} μg ml^{-1}	RF	EC_{50} μg ml^{-1}	RF	EC_{50} μg ml^{-1}	RF
$ViRM_1$	0.037	7.4	0.105	6.6	3.947	1.2
$ViRM_2$	0.069	13.8	0.148	9.3	4.125	1.2
$ViRM_3$	0.110	22	0.209	13.1	3.522	1.0
$ViRM_4$	0.041	8.2	0.125	7.8	4.309	1.3
$ViRM_5$	0.031	6.2	0.091	5.7	5.617	1.7
$ViBC_1$	0.148	29.6	0.362	22.6	4.159	1.2
$ViBC_2$	0.059	11.8	0.162	10.1	5.205	1.5
$ViBC_3$	0.054	10.8	0.153	9.6	3.712	1.1
$ViBC_4$	0.113	22.6	0.258	16.1	4.156	1.2
$ViBC_5$	0.038	7.6	0.081	5.1	3.360	1.0
$ViZB_1$	0.007	1.4	0.016	1.0	5.014	1.5
$ViZB_2$	0.005	1	0.019	1.2	5.141	1.5

In vivo tests. Flusilazole at all tested concentrations, showed excellent efficacy in prevention of disease caused by the sensitive isolate $ViZB_1$ (97.6–100%) (Table 5). When used at the concentration of 25 mg l^{-1}, which is equivalent to the commercially used concentration, flusilazole showed practically negligible efficacy (7.4%), when used on a resistant isolate $ViBC_1$. Almost the same efficacy (9.1%) was recorded when flusilazole was used at the concentration of 35 mg l^{-1}. When the initial concentration was doubled, the efficacy was low as well (25%). Somewhat greater, but from the practical standpoint, inadequate efficacy (66.3%), was observed when flusilazole was used at the concentration of 80 mg l^{-1}.

Difenoconazole completely prevented the disease development when used on a susceptible isolate $ViZB_1$ (Table 6). A negligible efficacy of 8.6% was recorded when difenoconazole at the concentration of 30 mg l^{-1} was used on the resistant isolate $ViBC_1$. Even when the concentration was increased to 45 mg l^{-1}, the efficacy was low (33.2%), and when the concentration was increased to 75 mg l^{-1}, the efficacy recorded was 65.7%. When used at the concentration of 100 mg l^{-1}, difenoconazole showed 81.9% efficacy, which could be considered satisfactory, although at that concentration, it also showed phytotoxic effects. The development of apple seedlings was completely stopped, they did not form new leaves, and the size of a stem did not change from the time the fungicide was applied, which confirms findings of Buchenauer and Röhner (1981) and Fletcher and Hofstra (1988), regarding a stunting effect of DMI fungicides.

Both demethylation inhibitor fungicides, when used in concentrations 2–3 times greater than the commercially recommended ones, showed inadequate efficacy when applied on the isolate $ViBC_1$, which was regarded as resistant to flusilazole in *in vitro* tests; this confirms the correlation between the two methods. The obtained results concur with the findings by Köller et al. (1997) and Kunz et al. (1997).

The results presented above, however, do not mean that by doubling the concentration of the fungicides in the field conditions, the resistant populations of *V. inaequalis* would be controlled. This is supported by the fact that the fungicide application in *in vivo* tests was conducted one day before the inoculation or 48 h before infection, which provides optimal conditions for the action of a fungicide, which rarely happens in the field conditions. Other important differences between laboratory and field conditions are the factors that influence degradation of the active substance (rain, UV radiation, wind, etc.), which do not exist in laboratory conditions.

Captan showed a complete control of both wild-type and isolate $ViBC_1$ (resistant to DMI fungicides) of *V. inaequalis*. Because the fungicide was applied before the infection occurred, knowing that captan should be used preventatively, the conditions for its efficacy were favourable. The results showed that there is no difference between DMI resistant and DMI sensitive isolates regarding sensitivity to captan (Table 7).

Table 5. Disease severity and efficacy of different concentrations of flusilazole

Flusilazole mg l^{-1}	Isolate			
	sensitive ($ViZB_1$)		resistant ($ViBC_1$)	
	severity %	efficacy %	severity %	efficacy %
0.0	87.4	–	81.6	–
25	2.1	97.6	75.6	7.4
35	0.0	100	74.1	9.1
50	0.0	100	61.2	25.0
80	0.0	100	27.5	66.3

Table 6. Disease severity and efficacy of different concentrations of difenoconazole

Difenoconazole mg l^{-1}	Isolate			
	sensitive ($ViZB_1$)		resistant ($ViBC_1$)	
	severity %	efficacy %	severity %	efficacy %
0.0	87.4	–	81.6	–
30	0.0	100	62.2	8.6
45	0.0	100	54.5	33.2
75	0.0	100	28.0	65.7
100	0.0	100	14.8	81.9

Table 7. Disease severity and efficacy of different concentrations of captan

Captan mg l^{-1}	Isolate			
	sensitive ($ViZB_1$)		resistant ($ViBC_1$)	
	severity %	efficacy %	severity %	efficacy %
0.0	87.4	–	81.6	–
1000	0.0	100	0.0	100
1500	0.0	100	0.0	100

Conclusions

1. In field experiments conducted for two years, low efficacy of demethylaton inhibiting fungicides flusilazole and difenoconazole was established, whereas the multi-site fungicide captan showed high efficacy in apple scab control.

2. In laboratory tests, isolates of *V. inaequalis* originated from experimental and commercial orchards, where DMI fungicides had been used intensively for more than ten years, showed significantly lower sensitivity to flusilazole and difenoconazole than isolates originated from orchard where those fungicides had never been used. All isolates tested in this study, showed very similar sensitivity to fungicide captan.

3. Using different methods, it was demonstrated in this study that after multiple-year use of DMI fungicides, *V. inaequalis* developed resistance to that fungicide group.

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Venturia inaequalis atsparumas demetiliaciją slopinantiems (DMI) fungicidams

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Santrauka

Venturia inaequalis jautrumas demetiliaciją slopinantiems (DMI) fungicidams tirtas lauko ir laboratorinėmis sąlygomis. Lauko bandymai vykdyti 2008–2008 m., jų metu tirtas triazolų cheminės grupės fungicidų difenokonazolo ir flusilazolo efektyvumas, palyginti su kaptano, priklausančio *Phtalamide* cheminei grupei, efektyvumu. Monosporinių *V. inaequalis* izoliatų iš vietovių su skirtingu fungicidų naudojimo intensyvumu jautrumas fungicidams buvo nustatytas laboratorijoje. Nustatytas grybienos augimo ant mitybos terpės slopinimas su flusilazolu, difenokonazolu ir kaptanu, o jautrumo fungicidams parametrai (EC_{50} vertės) nustatyti naudojant *probit* analizę. Grybo izoliatų jautrumas *in vivo* buvo tirtas ant obelų daigų, taikant inokuliavimo metodą.

Lauko bandymų metu nustatytas mažas abiejų DMI fungicidų – difenokonazolo bei flusilazolo – efektyvumas ir didelis captano efektyvumas. EC_{50} vertės flusilazolui svyravo nuo 0.005 iki 0.148 μg ml^{-1}, difenokonazolui – nuo 0.016 iki 0.362 μg ml^{-1}. *V. inaequalis* izoliatai iš sodų, kuriuose DMI fungicidai niekada nenaudoti, buvo vidutiniškai jautrūs visiems fungicidams, o izoliatai iš eksperimentinių ir prekinių sodų buvo atsparūs ir flusilazolui, ir difenokonazolui. Visų tirtų izoliatų jautrumas kaptanui buvo panašus.

Reikšminiai žodžiai: *Venturia inaequalis*, flusilazolas, difenokonazolas, efektyvumas, jautrumas, atsparumas.